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*Published in:*
Brain-research

*DOI:*
10.1016/S0006-8993(02)03703-4



*Publication date:*
2002

ELSEVIER

Brain Research 958 (2002) 463–467

BRAIN RESEARCH

www.elsevier.com/locate/brainres

Short communication

# Increase in somatostatin immunoreactivity in the suprachiasmatic nucleus of aged Wistar rats

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Accepted 24 September 2002

**Abstract**

Decreased immunoreactivity has been reported for several neuropeptides in the aged suprachiasmatic nucleus (SCN). We compared somatostatin (SS) and substance P (SP) immunoreactivity (ir) in aged (20–26 months) and young (6 months) Wistar rats. The old rat SCN revealed a significant increase in SSir (2.6-fold) and SPir. The results show that not all SCN–neuropeptidergic systems decline with age, and suggest a specific age-related role for SS in the SCN.


*Theme:* Neural basis of behavior

*Topic:* Aging

*Keywords:* Aging; Suprachiasmatic nucleus; Somatostatin; Substance P; Immunocytochemistry

The suprachiasmatic nucleus (SCN) of the hypothalamus is the main circadian pacemaker in mammals, driving circadian rhythms in behaviour and physiological functions. The rat SCN is divided anatomically into a ventrolateral (VL), an intermediate (IM) and a dorsomedial (DM) part, or, as more recently proposed [9] a core and a shell, corresponding to the ventral input site with large neurones and the dorsal region with smaller neurones, respectively. Neurones synthesising vasoactive intestinal peptide (VIP) and gastrin releasing peptide (GRP) are located in the core of the SCN, whereas vasopressin (AVP) neurones are found in the shell. Somatostatin (SS) producing neurones have been demonstrated in the core and shell [18], and substance P (SP) neurones (but especially fibres) in the core [16]. Part of SS and SP peptides are co-localised [13]. The VL SCN is involved in the entrainment of the pacemaker to light [15], and projects within the SCN to the shell, and outside the SCN mainly through VIPergic fibres. The shell, possibly involved in the regulation of overt rhythms, projects outside the SCN through AVPergic fibres [20], and within the SCN through SS fibres [3].

Ageing of the circadian system has been associated with amplitude reduction of circadian rhythms, fragmented rhythms, higher day-to-day rhythm variability, shortening of period length and altered responses to light [19,22]. The physiological basis for these age-related changes in circadian rhythms may be (partly) found in altered SCN neuropeptide organisation. Loss of AVPir [11,21] and VIPir [2] has been shown in aged rodents. Since the effect of age on SS and SP peptide expression has not been studied, we compared SS and SPir in the SCN of aged and young Wistar rats.

Subjects were nine young adult (6 months) and nine old (20–26 months) male Wistar rats obtained from the breeding facilities of the department of Animal Physiology, University of Groningen, The Netherlands. The animals were kept on a 12:12 h reversed light–dark cycle, with lights off at 09:00. Rats were individually housed, with food (Hope Farms standard rodent pellets) and water available ad libitum. From earlier observations in our laboratory on rats of the same strain and age, it was clear that the circadian system showed typical age-related changes in behavioural parameters, such as shortening of

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0006-8993/02/$ – see front matter 
PII: S0006-8993(02)03703-4

the freerunning period, fragmented rhythms, and decreased amplitudes. All young rats were sacrificed between 12:00 and 13:00. Of the old rats, five were sacrificed at 09:30, and four between 11:30 and 13:30. A circadian rhythm is present in SS peptide content [14], with a peak before mid-subjective day. Small divergences in the timing of peak value between young and aged animals (altered phase relationship to the Zeitgeber) could severely influence the outcome. For this reason, rats were sacrificed during the dark phase to ensure basal levels of SS content. All rats were deeply anaesthetised with 6% sodium pentobarbital (300 mg/kg). The brains were quickly removed and immersed in 4% paraformaldehyde (PAF) in 0.1 M phosphate buffer (PB) for 8 h. Before cutting, the brains were cryoprotected in 30% buffered sucrose solution at 4 °C for 48 h and, subsequently, cut coronally on a cryostat into 25-μm sections.

Free floating brain sections were preincubated with 0.2% peroxidase for 30 min at room temperature (RT) followed by overnight incubation at RT with normal goat serum or normal sheep serum (1%) and the primary antibody rabbit-anti-SS (directed against the COOH terminal portion of S-14; specificity of this antibody was confirmed by preabsorption experiments) 1:2000 (kindly donated by Dr. C. Rougeot, Pasteur Institute, Paris, France), rabbit-anti-SP 1:2000 (Biotrend, Germany) or mouse-anti-AVP 1:500 (PS41, kindly supplied by Dr. H. Gainer, NIH, MD, USA). All sections for one marker were run in the same assay. Triton X-100 (0.5%) was added to all incubation steps. After rinsing, the sections were incubated with biotinylated goat-anti-rabbit IgG (Zymed 1:200; SS/SP) or biotinylated sheep-anti-mouse IgG (Amersham 1:200; AVP) for 2 h. After rinsing, they were exposed to streptavidin horseradish peroxidase (Zymed 1:200) in PBS for 2 h at RT. Sections were stained using the diaminobenzidine (DAB)–$H_2O_2$ reaction (30 mg DAB/100 ml PBS). They were mounted onto glass slides, and cover slips were sealed with mountant DPX. Omission of the primary antibody yielded immunonegative results.

For SS, sections from each rat were taken, containing the SCN at three rostro–caudal levels. AVP was analysed at the medial level only. The optical density (OD) of SS and AVP in the SCN was determined with the aid of a QUANTIMET-600 image analysis system. With every new slide measured, the OD of the coverslip plus glass slide plus DPX was subtracted from the picture. Relative OD was calculated as: $[(OD_{SCN} - OD_{background})/OD_{background}]$ to correct for background staining and allow between-section comparison. Settings were adjusted in such a way that all OD measurements fell within a linear trajectory. The lateral hypothalamus adjacent to the SCN, devoid of SS or AVP staining served as background region (non-specific staining). OD measurements for the right and left SCN were averaged. The medial preoptic area (MPO) was measured in sections just anterior to the frontal SCN as a control area for SS. The low staining intensities of SCN–SP made it unsuitable for OD quantification. Therefore, a blindly performed semiquantification of staining density was done, using the following categories: (1) 'ghost-image' (denser staining in areas surrounding the SCN); (2) no staining; (3) very light staining and (4) moderate staining.

The effects of age and SCN level were assessed by two-way ANOVA. In case of significant ($P<0.01$) $F$ values, posthoc multiple comparisons were made with a Bonferroni $t$-test. For vasopressin, the effect of age was determined with a two-tailed $t$-test. All values are displayed as average±standard error of the mean (S.E.M.). This study was carried out with the approval of the Groningen Animal Experiment Committee.

Results show that the SCN contained SSir neurones, as well as numerous ir boutons (Fig. 1A–F) in both young and aged rats. SSir neurones could be detected at all three rostrocaudal levels. SS neurones were scattered in the whole SCN (core and shell) at the rostral level. At the medial level they were primarily located in the intermediate zone, and more lateral (shell) at the caudal level. Large, clearly stained neurones were present in the periventricular nucleus along the third ventricle and in the medial preoptic nucleus. In the aged SCN, the level of SSir was clearly increased, both at the level of the cell bodies and the boutons. Very few SPir fibres or neurones could be detected in either young or aged rats (Fig. 1G–H) at all SCN levels, in contrast to surrounding hypothalamic tissue.

Quantification of SSir by means of optical density (OD) measurements revealed no staining differences within the group of aged rats due to the time of sacrifice (09:30 vs. 11:30–13:30; 0.97±0.15 and 1.15±0.14, respectively). Therefore, these data were pooled. Fig. 2 shows SS OD measurements of old versus young rats at three rostrocaudal levels. Statistical testing confirmed a significant increase in SSir with ageing at all SCN levels (two-way ANOVA, $P<0.001$). There was a significant interaction between age and SCN level on SSir (two-way ANOVA, $P=0.005$). Interestingly, old rats had a significant rostrocaudal gradient from high to low SSir ($P<0.001$). This in contrast to young rats, where SSir in the medial SCN was slightly, but significantly higher than in the caudal part ($P<0.001$). To check whether the increase in SSir was SCN specific, the OD of the MPO just anterior to the SCN was measured. This revealed no difference between young and aged rats (0.30±0.02 and 0.23±0.02, respectively). A ranking test on the semiquantification of SPir revealed that the aged group had a significantly higher ranking (Mann–Whitney $U$, $P=0.003$).

AVP-positive neurones were present in the shell of the SCN in both young and aged rats. OD measurements (Fig. 2) were performed in the medial part of the SCN; this revealed a significant decline in AVPir with age ($t$-test, $P=0.004$).

Here we report a remarkable, SCN-specific increase of SSir with ageing. To our knowledge, there is only one

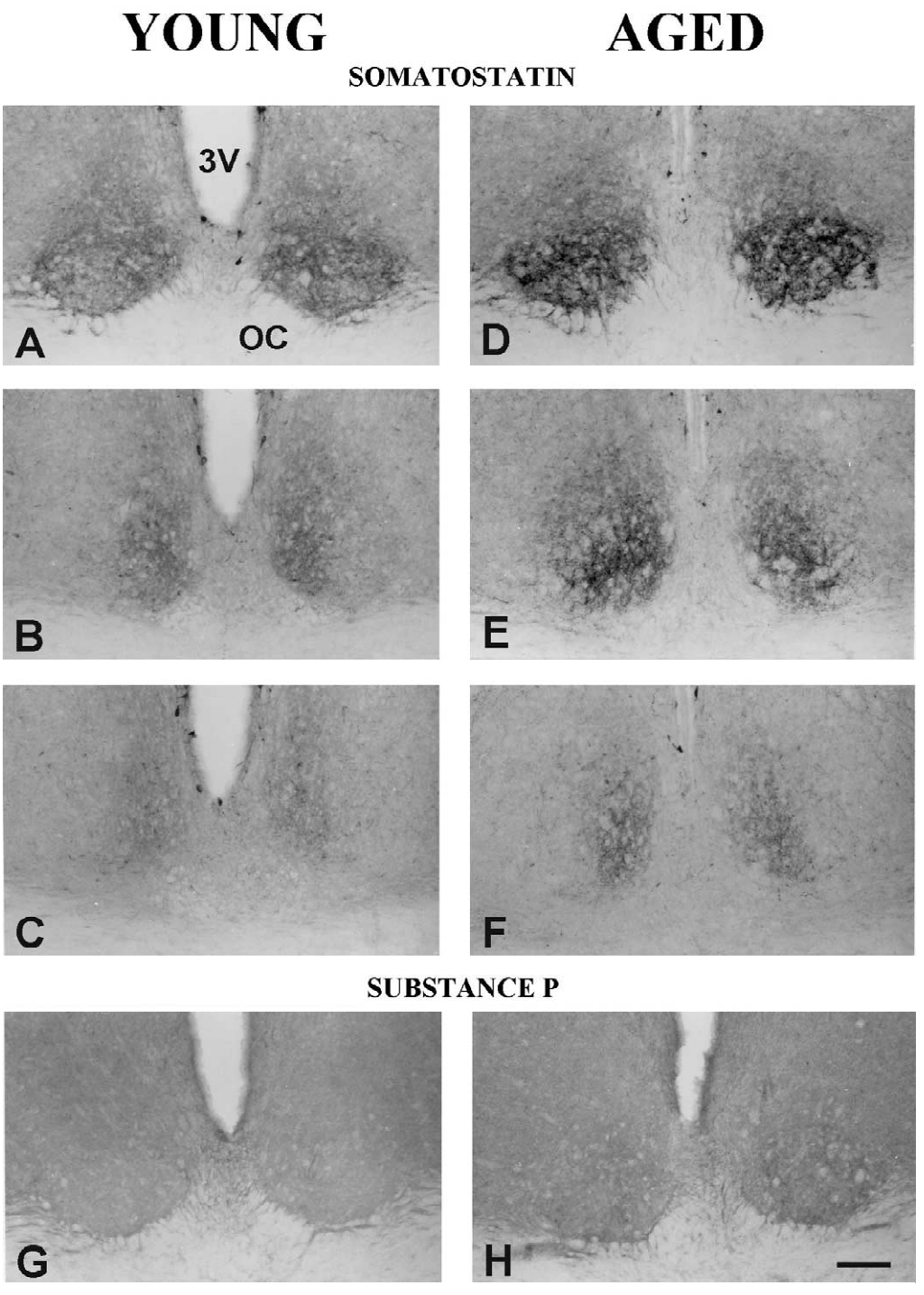


Fig. 1. Neuropeptide immunoreactivity in the young (left panels; A, B, C and G) and the old (right panels; D, E, F and H) Wistar rat SCN, showing representative examples of SSir at a rostral (A,D), medial (B,E) and caudal (C,F) level, and of SPir at the medial (G,H) level. 3V: third ventricle, OC: optic chiasm, scale bar = 120 μm.

study investigating the effect of age on SS in the SCN, at the level of SS mRNA in Syrian hamsters. No significant effect of age was found in this study [5]. The circadian profile of SS mRNA expression is also absent in these hamsters, in contrast to findings in rats [17]. Aging effects on SCN neuropeptide expression might well be species specific, as is the circadian profile of peptide expression in the SCN. Assessing a circadian profile of SSir in young and aged rats would be a logical next step in this study. The decrease of AVPir with age is consistent with earlier reports [11,21] and makes it unlikely that the increase is due to immersion fixation procedures.

The distribution of the SSir neurones within the SCN along the rostrocaudal axis is in accordance with studies on

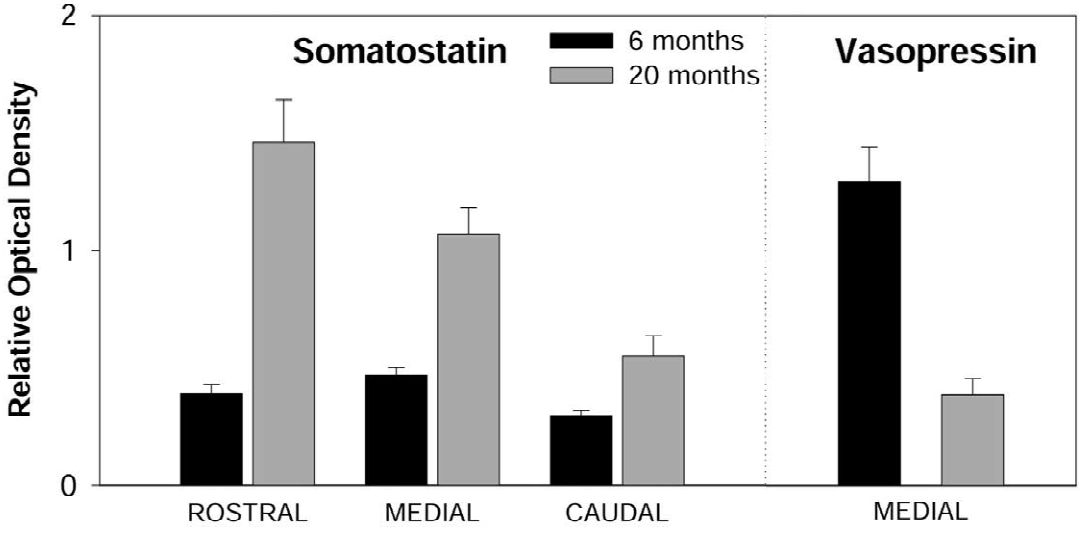


Fig. 2. Relative optical density measurements for SSir at three rostrocaudal levels of the SCN and AVPir at the medial SCN.

SS in Wistar rats [4,13,18]. An intriguing rostrocaudal gradient in SSir in the SCN was found in old rats. This suggests that the topographical distribution of SSir becomes more pronounced with age due to functional alterations, predominantly at the rostral level.

The few observed SPir neurones and fibres are in accordance with previous studies in rats [10,20], although other researchers have reported higher degrees of stainable SP in the SCN [16]. The slight increase in SPir that we found in old rats could be consistent with the finding that SS and SP are largely co-localised [13].

We can only speculate on possible functions of this increased SSir in old individuals. Increased ir by itself can reflect a raise in the production of SS peptide, or could, alternatively, be due to accumulation of nonreleased peptide in neurones of aged rats. SS neurones have a high degree of connectivity with other SCN neurones, suggesting an important modulatory role. In view of the reported role for SS in phase shifting [6,8], this could lead to interesting concepts, such as accumulation of SS in SCN cell bodies leading to impairments in the ability to adjust to altered LD situations (e.g. resynchronisation; phase responses to light pulses). Besides SS, SP is also able to reset the phase of the clock [12]. Moreover, the findings that SS fibres synapse on VIP and AVP neurones [3] and that SS receptors are predominant in the SCN [1] suggest that SS regulates other peptidergic neurones. In fact, SS has an inhibitory modulating role on VIP rhythmicity [7]. Increase in SSir due to elevated production could explain the observed VIP decrease with ageing. If, on the other hand, enhanced levels of SSir reflect a release ‘deficit’, this would lead to a reduction of inhibitory actions. In summary, the SCN-specific age-related increase in SSir might be a causal factor in alterations of circadian clock-function in the aged rat.

## Acknowledgements

The authors thank Dr. R. Hut and Professor Dr. S. Daan for critically reading the manuscript.

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